Volume 257, number 2, 307-310 FEB 07776 November 1989

A chemically transformed rat fibroblast cell line expresses high levels of oncomodulin

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Received 11 September 1989

Chemically (by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) treated rat fibroblasts (T14c) exhibited growth characteristics and a morphology typical for transformed cells and markedly different from untreated, parental cells. In contrast to untransformed cells, T14c fibroblasts produced significant levels of oncomodulin mRNA as analyzed on Northern blots even when compared to rat Morris hepatomas, the richest source of oncomodulin known so far. The levels of transcripts for both calmodulin and oncomodulin in T14c cells were higher in log phase growth as compared to confluent stages. The T14c model system may be useful in the elucidation of mechanisms involved in the regulation of oncomodulin synthesis.

Chemical transformation; Fibroblast; Ca^{2+}-binding protein; Oncomodulin; Calmodulin

1. INTRODUCTION

OM is an oncodevelopmental calcium-binding protein structurally related to parvalbumin. Its expression is restricted to tumor cells and the placental cytotrophoblasts of rodents and man [1-3]. The potential of OM to activate enzymes in an analogous fashion to the multifunctional calcium-binding protein CAM is controversial [4,5]. Recently, it was proposed that the active molecule could be a cysteine linked OM dimer [6]. So far, the in vivo function of OM is not known. Based on the identification of a high sequence homology of the OM mRNA leader sequence and endogenous retroviral LTRs and the isolation of such a putative regulatory element from a rat genomic library, we recently proposed that OM expression might be controlled by an LTR promoter [7]. Analysis of the rat OM gene including promoter sequences showed that a solo LTR is located adjacent to the OM transcription start site [8].

OM was originally purified from rat Morris hepatomas, which have remained the main source for the isolation of this protein [9]. One of the factors limiting the study of molecular mechanisms involved in the regulation of OM synthesis and in the elucidation of consequences of OM expression has been the lack of an established cell line producing high amounts of this protein. Therefore, we transformed rat fibroblasts (in vivo by MNNG) using the air pouch method and identified several clones that stably express OM at high rates [10]. In the present study we analyzed the morphology and growth characteristics of one such transformed cell line (T14c) in comparison to normal, untreated pouch fibroblasts. In addition, we measured OM and CAM mRNA levels during growth of both cell types and in Morris hepatoma solid tumors.

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Abbreviations: CAM, calmodulin; OM, oncomudolin; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; LTR, long terminal repeat

2. MATERIALS AND METHODS

2.1. *Cell culture and tissues*

Both normal and transformed granuloma pouch fibroblasts, first described by Sommer and Heizmann [10], were cultured in DMEM (Gibco, Scotland) supplemented with 10% fetal calf serum (Amimed, Switzerland) and 50 μg/ml gentamycin (Seromed, FRG) followed by incubation in a humid atmosphere of 8% CO_2, 5% O_2 at 36°C. Buffalo rats carrying Morris hepatomas were obtained from Dr Slaughter, Howard University, Washington DC, USA. Tumors of 30-50 g were excised, freed from necrotic material and stored at −80°C before RNA extraction.

2.2. *Growth curve*

Cell monolayers from stock cultures were detached by 0.25% trypsin (Gibco, Scotland) and resuspended in growth medium. Cells were then seeded at an initial density of 10^6 cells per 20 ml medium in 14 cm diameter tissue culture dishes (Nunc, Denmark) and incubated at standard conditions with a change of medium every 48 h. At 12-h intervals, cells were trypsinized into growth medium and cell numbers were determined using a Coulter particle counter (Coulter Electronics, France).

2.3. *RNA isolation*

Cells were treated to establish growth curves and the final cell suspension was centrifuged at 500 × *g* for 5 min at 4°C and washed three times with cold phosphate-buffered saline. Cell pellets were

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00145793/89/$3.50

stored at −80°C till used to isolate total RNA as described in [11]. Average RNA yields were 100–150 $\mu g/10^7$ cells as determined by measuring optical density at 260 nm. Total RNA was similarly isolated from Buffalo rat brains and Morris hepatoma solid tumors.

2.4. *Northern blots and dot blots*

Equal amounts of total RNA from various rat tissues or from normal and transformed cell cultures at different degrees of confluence were run on horizontal gels containing 1.4% agarose and 6% formaldehyde, followed by transfer onto Biodyne-A membranes (Pall, Glen Cove, NY). Dot blotting was performed as described in [12]. Blots were hybridized to ^{32}P-labeled CAM or OM cDNA and washed at high stringency. Autoradiography was performed at −80°C using Kodak X-AR films with two screens.

2.5. *Labeling of cDNA probes*

OM cDNA probe 0MpGEMθ 3+700 derived from a placental cDNA λgt11 library [13] and CAM cDNA probe CAM 22 [14] were labeled according to [15], resulting in a specific activity of $1–2 \times 10^9$ cpm/μg, and separated from free radioactivity by passage over a Sephadex G-50 column in 20 mM NaCl, 20 mM Tris-HCl, 2 mM EDTA, and 0.25% SDS (pH 7.5).

3. RESULTS AND DISCUSSION

3.1. *Cell morphology and growth*

Normal granuloma pouch cells in log phase growth appear large and flat. In contrast, neoplastic T14c cells exhibit a finer, more elongated morphology and are much smaller (fig.1). After an initial lag phase of 12–24 h, both cell types doubled in approximately 24 h. Under the conditions used for the growth curve, normal cells were contact-inhibited after 48 h, resulting in a strongly adherent monolayer, whereas T14c cells grew exponentially and over each other for about 120 h (fig.2).

3.2. *Expression of calmodulin and oncomodulin mRNA*

Northern blot analysis of total RNA revealed no detectable OM signal in rat brain or normal rat fibroblasts, whether or not subjected to pouch treatment (fig.3). Both T14c cells and Morris hepatomas contain, however, a single OM transcript of about 600–700 nucleotides as determined by rRNA internal standards. Densitometric evaluation of dot blots showed approximately 4-fold higher OM mRNA steady state levels in Morris hepatoma as compared to T14c cells. T14c appears to be the cell line with the highest OM synthesis so far reported. Levels of transcripts for both CAM and OM were significantly higher (in the range of 100%) in log phase growth as compared to confluent stages

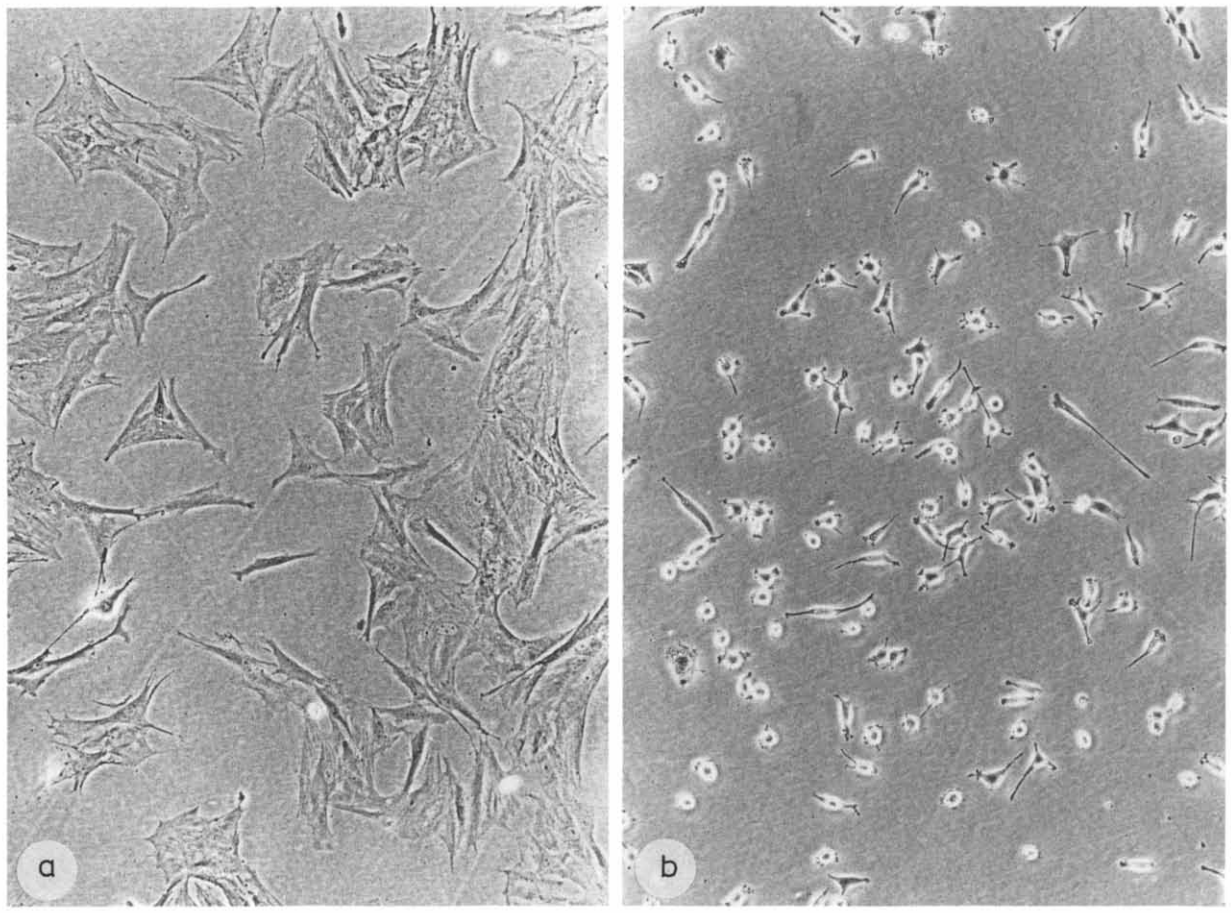

Fig.1. Phase contrast microscopy of normal rat fibroblasts (a) and chemically transformed fibroblasts, T14c (b) in log phase growth. The transformed phenotype reveals a spindle-shaped morphology while mitotic phase cells are rounded.

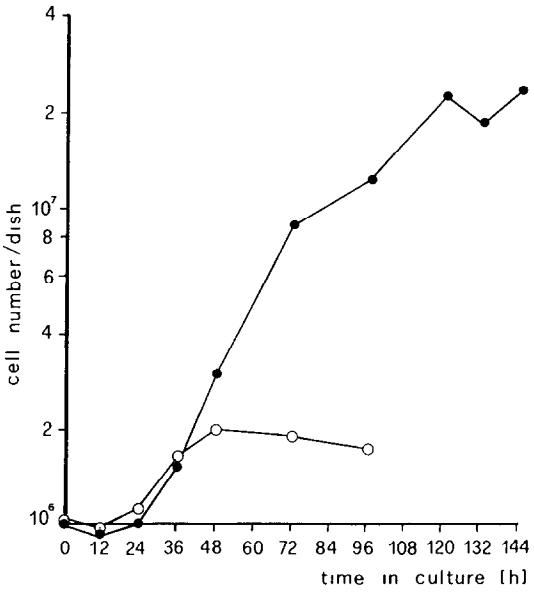

Fig.2. Comparison of growth curves for T14c (closed circles) and normal control fibroblasts (open circles).

(fig.4). The amount of OM mRNA appeared to be several-fold greater than that of CAM mRNA, although it must be emphasized that the signal intensities on Northern blots cannot be directly compared due to different lengths and species origin of the probes. In the

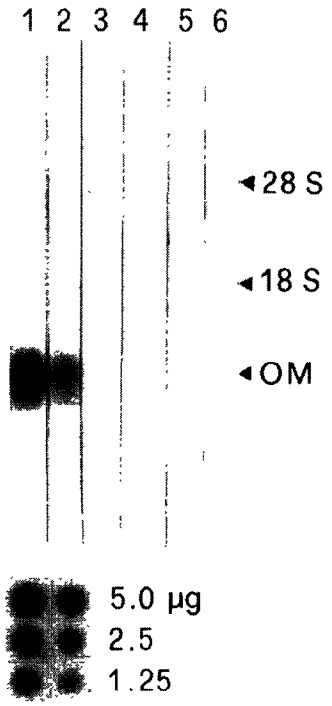

Fig.3. Northern and dot blot analyses of various rat cell cultures and tissues hybridized to ^{32}P-OM cDNA and ^{32}P-CAM cDNA. 10 µg of total RNA were electrophoresed and blotted. Lanes: 1, Morris hepatoma; 2, T14c; 3, fibroblasts, pouch activated; 4, fibroblasts, without pouch treatment; 5, rat brain; 6, tRNA. Dot blots are shown for semi-quantitative comparison. Autoradiography was for 2 days with intensifying screens.

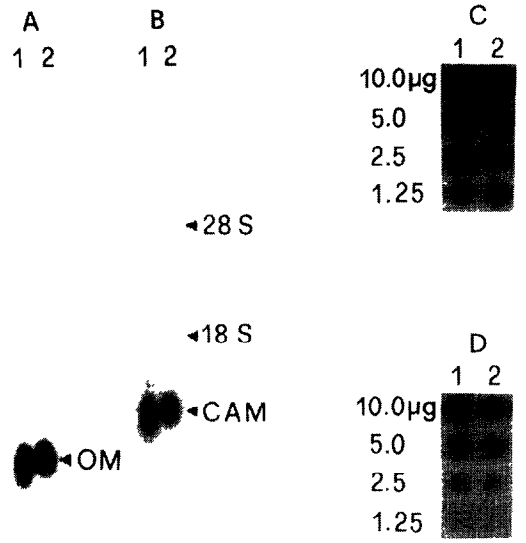

Fig.4. Northern and dot blots of T14c cells at different degrees of confluence hybridized to ^{32}P-OM cDNA and ^{32}P-CAM cDNA. 30 µg of total RNA were used for Northern blots. Autoradiography was for 24 h for A and 72 h for B, C and D, with intensifying screens. Dot blots are shown for semi-quantitative comparison. A and C: hybridization with ^{32}P-OM cDNA; B and D: hybridization with ^{32}P-CAM cDNA. Lanes 1: 50% confluence; lanes 2: 100% confluence.

rat genome, three bona fide CAM genes encode the same protein [16]. We found four CAM signals on Northern blots of T14c RNA. The major signal, about 1400 nucleotides in length, corresponds to the mRNA transcribed from CAM II, which is the primary transcript found in several normal tissues and all tumors of rats and mice screened so far [17]. This transcript was markedly reduced after T14c cells entered the plateau stage of growth. No significant variation in the ratio of signal intensities of the different transcripts were found in cells at different stages of confluence and between normal and transformed cells.

The high level of OM expression found in T14c cells opens up new possibilities in the study of the regulation of OM synthesis. Though the levels of both CAM and OM transcripts were progressively reduced with increasing time in culture, it is not yet possible to correlate these changes to events in the cell cycle of transformed rat fibroblasts. In contrast to OM, CAM is found in normal cells and at elevated levels in rapidly proliferating cells [18]. It is known that the progression of cells through the G_1 phase is critically dependent upon CAM expression. Its specific function in the G_1-S transition in mouse C-127 cells was recently demonstrated [19,20] and CAM has been shown to be essential for yeast cell cycle progression [21]. It has been reported that OM can substitute for CAM in stimulating DNA synthesis in Ca^{2+}-deprived cells [22]. In addition, it was shown that OM can replace CAM to some degree in its activation of heart phosphodiesterase [4]. A role for OM similar to CAM with respect to cell cycle progres-

sion and tumor cell proliferation has not been reported so far. No experimental data are available concerning cell phase specific expression of OM. Therefore, we plan to examine the possible cell cycle association of OM expression using the transformed granuloma pouch cell system as a model.

Acknowledgements: The Swiss National Science Foundation (Grants 3.634-0.87 and 3.139-0.88), the Swiss Cancer League (FOR 337.87.2) and the Kanton Zurich Anti-Cancer League are acknowledged for financial support.

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